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Progress Report
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P.I. H. Shaw Warren M.D.
Massachusetts General Hospital
Boston, MA 02114

I. Work Summary

At the time of our last report (Oct 30, 1994) we were frustrated by several problems that resulted in the lack of a single amino acid sequence mimicking the active site of LPS binding protein (LBP). We had narrowed the sequence to a region between 86 and 106. By varying the amino terminal using overlapping sequences, we determined that the smallest peptides that retained LPS binding activity terminated with residue 86. Two questions remained concerning the C terminal. First, the importance of amino acids at positions 103-106 were in doubt. Second, most of our earlier peptides had been generated with a terminal cysteine on the C terminal to facilitate coupling to IgG. We were concerned that some of these peptides could have spontaneously dimerized, raising the possibility that some of the binding activity was due to dimers rather than monomers of peptides.

At this point we have shown that we can create LBP-IgG conjugates that bind LPS in buffer with high affinity. These same peptides block the action of LPS in assays of cytokine release. The next stage of the project is to vary aspects of the coupling (number of peptide copies/IgG, linker molecule) for desirable properties and to scale up for animal studies. In order to avoid time consuming and expensive experiments in several directions at once, a **single** peptide sequence is needed. We therefore focused our time over the last trimester in identifying our single best LBP peptide. As noted in our last progress summary, we simultaneously started to study the stability and properties peptide-IgG conjugates based on another LPS binding peptide (CAP18).

A. Progress on Specific Aim #1

As of the last progress report, we had designed an experiment to narrow down the optimum sequence for the carboxy terminal of the LBP peptide that we would focus on. Accordingly we synthesized the following peptides to establish the importance of amino acids 103-106, to study the importance of dimerization, and to evaluate if the reversed sequence had any activity:

LBP86-102C
LBPC102-86 (reversed sequence)
LBP86-106C
LBP86-102-106-105-104-103C (LBP86-102 plus last four amino acids of 86-106 in reversed order)

LBP86-102C and LBPC102-86 were reduced, deliberately dimerized, and treated by a blocking reagent (iodoacetamide) on the carboxy cysteine to prevent dimerization. The reversed sequences were generated to provide identical hydrophobicity and charge but in a different order. We tested the resultant peptides for ability to bind LPS in the slot blot assay and the ability to block binding of LPS to CD14 transfected CHO cells in LBP containing human serum as assessed by FACS. This latter assay assesses the ability of the peptides to block the LBP-CD14 axis that is necessary for macrophage activation and depends upon native LBP. Active peptides appear to block by interfering with LPS-native LBP interactions. The results of this experiment, which were somewhat complex, suggested that:

1. LBP86-102C and LBP86-106C bound LPS well and blocked LPS binding to CD14. LBP86-102C dimerized and reduced seemed to have similar activity, although the activity of all of the peptides with this sequence was somewhat less than with several prior batches of peptides. LBP86-106C had more consistent and slightly higher activity.
2. LBPC102-86 also bound and blocked, but optimal activity was seen in the dimerized form. Blocking the cysteine or reducing the peptide prior to testing resulted in a moderate loss of activity.
3. LBP86-102-106-105-104-103C bound LPS and blocked binding to CD14 almost as well as LBP86-106C.

Based on this information, we believe that LBP86-102 is the smallest sequence that binds LPS and blocks its interaction with LBP and CD14. However, because LBP86-106C blocked more consistently and had slightly higher activity, we believe that it will be a better choice for coupling to IgG. In addition, the four "extra" amino acids (103-106) will presumeably lead to less interference with the 100-102 AA part of the sequence after coupling. We believe this part of the peptide is important for activity. Thus, we have selected LBP86-106C as our best candidate for further coupling experiments. To doubly confirm that the cysteine is not playing a role in the binding for the purposes of publication, we are in the process of synthesizing this sequence +/- the cysteine for analagous studies. In the meanwhile, we are generating a large batch of 86-106C for coupling.

B. Progress on Specific Aim #2

Because of unexpectedly positive results on the project with LBP early in the first year, we have focused primarily on the LPS binding sequence of LBP. We are now designing and synthesizing peptides that correspond to the presumed LPS binding site of BPI (based on sequences homologous to our LBP peptides and the published work of others studying BPI. We are just beginning this specific aim and have no new data yet.

C. Progress on Specific Aim #3

This specific aim is well underway. As noted in earlier reports, we have settled on using human IgG prepared for intravenous injection for the bulk of our experiments because it is easily available in large quantities and is pyrogen-free. Until very recently, we concentrated on preparations IgG conjugates based on three peptides: hLBP76-102, hLBP86-102, and CAP18. As noted above, we chose the two hLBP peptides based on their activity, but had not earlier settled on a final peptide with which to scale up. We systematically

included the CAP18 peptide for comparison because it also binds LPS with high affinity. Initial conjugates with all three peptides bind LPS extremely well in aqueous buffers as assessed by RIA. As noted in our last report, an issue that has emerged that we believe is crucial for the further development of the conjugates is the ability of the conjugates to bind **in blood** (as opposed to buffer). Accordingly, as initially planned, we developed a magnetic bead assay with rabbit anti-hIgG covalently coupled to the beads that allows us to capture the peptide-hIgG conjugates in rabbit blood. By adding radiolabeled LPS to the blood, adding the conjugates, and then the capturing immunobeads, we have been able to compare the ability of the conjugates to bind LPS in whole blood. By incubating the conjugates in the blood for varying times before the addition of the LPS we are able to assess the functional stability of the conjugates in blood. Early experiments (outlined in the Oct. 30, 1994 report) indicated that the CAP18 conjugates lost activity over time. We were able to improve on the stability of the conjugates by coupling with SMPT as opposed to SPDP. SMPT is a heterobifunctional linker that has bulky groups that prevent reduction of the disulfide bond linking the peptide to the IgG. Even with SMPT, there was an initial rapid loss of activity over time, followed by a slow decay over 24 hours. Possibilities for this included that the conjugates were taken into WBC (perhaps a desirable property), or that the peptide was removed from the conjugate, or that there was proteolysis of the peptide itself. Most of these experiments were done with CAP18-hIgG conjugates because we had not settled on a final LBP peptide and we therefore did not have large quantities of any hLBP peptide to couple. A single experiment performed with an early batch of LBP86-102-IgG indicated that binding in blood was somewhat decreased compared to PBS, and that this effect can be partially compensated for by adding higher concentration of the conjugate. These experiments will need to be amplified and repeated in detail when newer peptides and conjugates are generated with optimum sequence and coupling techniques and with larger copies of peptide/IgG.

We have now worked out the technical aspects of coupling for three heterobifunctional couplers (SPDP, SMPT, and SMCC, a coupler that is apparently more stable than SPDP and SMPT in blood).

D. Progress on Specific Aim #4

A major goal of this specific aim was to develop appropriate assays to characterize the conjugates and to assess their ability to bind and neutralize LPS and bacteria. These assays are now all up and running and available to us.

One goal was to label peptides in order to accurately measure the number of peptides/IgG and to develop accurate structure function relationships. This information is crucial for the development of a final product, especially if it is eventually produced genetically. The unanticipated finding that the conjugates might not be stable in blood has increased our need to produce conjugates with labeled peptide so that we can directly measure the physical stability of the conjugates in blood. Conjugates radiolabeled in the peptide component would also facilitate pharmacokinetic experiments in mice and rabbits.

Over the last trimester, we produced a large batch (45 mg) of 14C-CAP18 that was labeled on the amino terminal of the peptide using 14C acetic anhydride while the peptide was still on the resin. Now that we have settled on the sequence of LBP as 86-106, we have started the synthesis of 14C-hLBP86-106C

using the same process. When this reagent is available, we will prepare SPDP, SMPT, and SMCC IgG conjugates with each.

It has become clear that it will be desirable to study the transport of the peptide-IgG conjugates into WBC in blood. It will be of equal importance to study the effect of the conjugates in transporting LPS and bacteria into WBC in blood. We are in the process of developing an assay of opsinophagocytosis in our laboratory. Ideally, we would like to study the ability of the conjugates to promote phagocytosis of whole intact bacteria and also released bacterial membrane blebs containing LPS. We are currently using radiolabeled chemically purified LPS. We believe that it will be far better to study the physicochemical form of native LPS that is released from bacteria into blood. We have therefore radiolabeled E. coli O111:B4 and S. typhimurium specifically in the LPS moiety by growing Gal-E mutants of each in 3H-galactose. Each of these strains form complete chain LPS under these conditions with almost all of the radiolabel incorporated into the LPS. We extracted the LPS of each and found that the specific activities were 53,000 and 4,600 CPM/ug, respectively. Incubation of the bacteria in rabbit, rat, and human sera causes about 30% of the radiolabeled LPS to be released. We plan to add WBC (purified over Ficoll) to serum containing 3H-LPS labeled bacteria to study phagocytosis and to compare the conjugates binding of purified LPS with LPS on bacteria or released from its surface in the form of blebs.

E. Progress on Specific Aims #5-7

As per our initial timetable, we have not started these animal experiments. To stay on our anticipated schedule, we will need to start experiments of pharmacokinetics of the conjugates by the middle of the year.

II. New knowledge since the last report

1. LBP86-106 appears to be the optimum sequence for LPS binding and blocking LPS-CD14 interactions (that are dependent upon native LBP). We have therefore selected this sequence for scaling up.

2. In a single experiment, an early preparation of LBP86-102-IgG coupled with SPDP bound tritiated LPS well in phosphate buffered saline but only modestly in 20% whole rabbit blood. These experiments need repeating and amplifying with new conjugates, the LBP86-106 sequence, peptides prepared with 14C-labeled peptide, and more stable heterobifunctional linkers.

III. Technical problems

We have had no technical problems during the last trimester.

IV. Future directions

We are essentially following our projected work plan very closely and are pretty much on schedule. We have now narrowed down the multiple different sequences that we had been working with LBP86-106, which should allow us to proceed more rapidly because we can focus only on this sequence. An important

goal for the next trimester will be to generate radiolabeled peptide and new conjugates with varying numbers of peptide/IgG with our three linkers. We will then test these for stability and function in blood. Specific goals for the next trimester are:

1. To prepare 14C-LBP86-106C.

2. To couple 14C-LBP86-106C to hIgG with varying copies of peptide (2,4,8-fold molar excess) per IgG using SPDP, SMPT, SMCC.

3. To compare LPS binding activities of these preparations in PBS and 20% whole rabbit blood using tritiated purified LPS and tritiated LPS in the form of membrane blebs of E. coli O111:B4 and S. typhimurium that have been prepared with Gal-E mutants.

4. To evaluate the stability of these preparations in rabbit blood and plasma.

5. To study if these conjugates are taken into WBC after incubation with blood.

6. To study if these conjugates increase the uptake of LPS into WBC after incubation of preformed 3H-LPS-14C-peptide-IgG conjugates in blood. If yes, to do the same experiment by adding the 3H-LPS to 14C-peptide-conjugate that is already present in blood.

7. To start to prepare and evaluate sequences of BPI that are homologous to LBP86-106 (specific aim #2)

V. Publications published or submitted in the last trimester

1. Kloczewiak M, Black KM, Loiselle P, Cavaillon JM, Wainwright N, Warren HS. Synthetic peptides that mimic the binding site of horseshoe crab anti-lipopolysaccharide factor. J. Infect. Dis. 170:1490-7, 1994.

2. Warren HS, Black KM, Loiselle PL. Range and distribution of natural antibodies to the O-antigen of lipopolysaccharides in human plasma. Submitted to J. Infect. Dis. (funded in part by the preceding Navy grant).

3. Warren HS, Cavaillon JM, Loiselle P, Ge Y, Black K, Zanzot E, Fitting C, Golenboch D, Vermeulen MW, Ezzell R, Kloczewiak M. Identification of a major LPS binding site of lipopolysaccharide binding protein. In preparation for submission.

4. Fletcher MA, Kloczewiak M, Loiselle P, Amato S, Black K, Warren HS. TALF peptide-immunoglobulin conjugates that bind lipopolysaccharide. In preparation for submission for "concise concept" section of J. Inf. Dis.

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